THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 276, No. 41, Issue of October 12, pp. 37761–37768, 2001
Printed in U.S.A.

Agonist-induced Signaling, Desensitization, and Internalization of a Phosphorylation-deficient AT_{1A} Angiotensin Receptor*

Received for publication, July 9, 2001, and in revised form, August 7, 2001
Published, JBC Papers in Press, August 8, 2001, DOI 10.1074/jbc.M106368200

J. Alberto Olivares-Reyes‡§, Roger D. Smith‡, László Hunyady¶‖, Bukhtiar H. Shah‡, and Kevin J. Catt‡ ‡‡**

From the ‡Endocrinology and Reproduction Research Branch, NICHD, National Institutes of Health, Bethesda, Maryland 20892 and the ¶Department of Physiology, Faculty of Medicine, Semmelweis University, H-1088 Budapest, Hungary

An analysis of the functional role of a diacidic motif (Asp^{236}-Asp^{237}) in the third intracellular loop of the AT_{1A} angiotensin II (Ang II) receptor (AT_1-R) revealed that substitution of both amino acids with alanine (DD-AA) or asparagine (DD-NN) residues diminished Ang II-induced receptor phosphorylation in COS-7 cells. However, Ang II-stimulated inositol phosphate production, mitogen-activated protein kinase, and AT_1 receptor desensitization and internalization were not significantly impaired. Overexpression of dominant negative G protein-coupled receptor kinase 2 ($GRK2^{K220M}$) decreased agonist-induced receptor phosphorylation by ~40%, but did not further reduce the impaired phosphorylation of DD-AA and DD-NN receptors. Inhibition of protein kinase C by bisindolylmaleimide reduced the phosphorylation of both the wild-type and the DD mutant receptors by ~30%. The inhibitory effects of $GRK2^{K220M}$ expression and protein kinase C inhibition by bisindolylmaleimide on agonist-induced phosphorylation were additive for the wild-type AT_1-R, but not for the DD mutant receptor. Agonist-induced internalization of the wild-type and DD mutant receptors was similar and was unaltered by coexpression of $GRK2^{K220M}$. These findings demonstrate that an acidic motif at position 236/237 in the third intracellular loop of the AT_1-R is required for optimal Ang II-induced phosphorylation of its carboxyl-terminal tail by GRKs. Furthermore, the properties of the DD mutant receptor suggest that not only Ang II-induced signaling, but also receptor desensitization and internalization, are independent of agonist-induced GRK-mediated phosphorylation of the AT_1 receptor.

The most intensively studied members of the superfamily of G protein-coupled receptors (GPCRs)[1] are rhodopsin and the β_2-adrenergic receptor (β_2-AR). Following agonist activation, G_s-mediated signaling by the β_2-AR is rapidly attenuated (or desensitized) and the receptor is internalized (or sequestered) into cells. In many GPCRs, these events result from phosphorylation of the agonist-activated receptors on serine/threonine residues by G protein-coupled receptor kinases (GRKs) and the consequent binding of β-arrestin proteins. Receptor-bound β-arrestins not only uncouple the β_2-AR from G_s and initiate desensitization, but also act as molecular adaptors in the endocytosis of β_2-ARs via clathrin-coated pits and promote intracellular signaling to mitogen-activated protein kinase cascades (reviewed in Refs. 1–4).

Agonist-induced desensitization occurs in almost all GPCRs, and is mediated by phosphorylation of the receptor by at least two classes of serine/threonine kinase: GRKs and second messenger-activated kinases, usually protein kinase A or C. Agonist-induced phosphorylation has been demonstrated in numerous GPCRs including the β_2-AR, m1 (5) and m2 (6) muscarinic, substance P (7), somatostatin (8), δ-opioid (9), endothelin (10), A_3 adenosine (11), V_2 vasopressin (12), and AT_1 and AT_2 angiotensin receptors (AT_1-R and AT_2-R) (13–17). Phosphorylation usually occurs at multiple serine or threonine residues, and in many cases these have been localized to specific regions of the receptors. In rhodopsin (18), the β_2-AR (19), the parathyroid hormone receptor (20), and the AT_1 receptor (15, 16), phosphorylation sites are located within serine/threonine-rich regions of the receptors' cytoplasmic tails. However, in the m2 muscarinic (21), α_{2A}-adrenergic (22), and α_{2B}-adrenergic (23) receptors, they are present in the third intracellular loop. Unlike second messenger-dependent kinases, which phosphorylate target proteins within well defined sequences (24), the consensus sites for GRK-mediated phosphorylation in GPCRs have not been unequivocally identified. However, the frequent proximity of acidic residues, and the acidotropic preferences of some GRKs for synthetic peptide substrates (25, 26) has suggested that Asp or Glu residues adjacent to Ser/Thr sites are required for GRK-mediated receptor phosphorylation (19, 22). Recent studies on the β-adrenergic receptor (19), the m_2 muscarinic receptor (27), the α_{2A}- and α_{2B}-adrenergic receptors (22, 23, 28), and the μ opioid receptor (29, 30) have shown that neighboring acidic residues are necessary for GRK-mediated phosphorylation.

The major agonist-induced phosphorylation sites of the rat AT_{1A}-R are located in a serine/threonine-rich segment (Ser^{326}–Thr^{338}) of the receptor's cytoplasmic tail (15, 16). However, the only potentially relevant acidic residues within the intracellu-

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by Pan American Fellowship NIH/CONACyt-979004.

‖ Supported by Hungarian Ministry of Culture and Education Grant FKFP-0318/1999 and Hungarian Science Foundation Grant OTKA T-032179.

** Supported in part by the Aga Khan University, Karachi, Pakistan.

‡‡ To whom correspondence should be addressed: Endocrinology and Reproduction Research Branch, NICHD, National Institutes of Health, Bldg. 49, Rm. 6A-36, 9000 Rockville Pike, Bethesda, MD 20892-4510. Tel.: 301-496-2136; Fax: 301-480-8010; E-mail: catt@helix.nih.gov.

[1] The abbreviations used are: GPCR, G protein-coupled receptor; AT_1-R, type 1 angiotensin receptor; AT_2-R, type 2 angiotensin receptor; β_2-AR, β_2-adrenergic receptor; BIM, bisindolylmaleimide; DD mutant, Asp^{236}-Asp^{237} mutant type 1 angiotensin receptor; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GRK, G protein-coupled receptor kinase; $GRK2^{K220M}$, dominant negative mutant of GRK2; HA, hemagglutinin; PKC, protein kinase C; PNGase F, peptide *N*-glycosidase F; Ang II, angiotensin II; BSA, bovine serum albumin; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.

lar domains of the AT_1-R are Asp^{236} and Asp^{237}, and these are located in the receptor's third intracellular loop rather than adjacent to the phosphorylation sites in the COOH-terminal cytoplasmic domain (31). Earlier studies have shown that individual mutations of these aspartate residues have no major effect on inositol phosphate signaling and internalization of the AT_1 receptor (32). However, in the present study, a more detailed analysis of this diacidic motif has demonstrated its importance in GRK-mediated phosphorylation of the receptor, and has raised questions about the general role of these kinases in the regulation of desensitization and endocytosis of GPCRs. This investigation of the functional importance of the diacidic motif has indicated its requirement in agonist-induced receptor phosphorylation of the AT_1-R and has also suggested that the majority of such phosphorylation is not essential for receptor desensitization and internalization.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from American Type Culture Collection. Phosphate-free DMEM, inositol-free DMEM, trypsin, antibiotic solutions, OptiMEM, and LipofectAMINE were from Life Technologies, Inc. Medium 199 was from Biofluids (Rockville, MD). Angiotensin II was from Peninsula Laboratories (Belmont, CA). ^{125}I-[Sar^1,Ile^8]Ang II and ^{125}I-Ang II were from Covance Laboratories (Vienna, VA), *myo*-[2-^{3}H]inositol was from Amersham Pharmacia Biotech, and $^{32}P_i$ was from ICN (Costa Mesa, CA). Protein A-agarose and bisindolylmaleimide I were all from Calbiochem (San Diego, CA). Peptide *N*-glycosidase F (*N*-glycanase) F (PNGase F; EC 3.5.1.52) was from Roche Molecular Biochemicals and from Glyco (Heyford, United Kingdom), and the HA.11 mouse monoclonal antibody was from BAbCo (Berkeley, CA).

Plasmids and Mutagenesis of the Rat AT_{1A} Receptor cDNA—The influenza HA epitope (YPYDVPDYA) was inserted after the codons of the amino-terminal first two amino acids (MA) into the cDNA of the rat AT_{1A} receptor subcloned into pcDNA3.1 (Invitrogen, San Diego, CA) as described previously (14). The expression vector pcDNA I-GRK2 dominant negative mutant ($GRK2^{K220M}$) was kindly donated by Dr. S. S. G. Ferguson (33). Site-directed mutagenesis was achieved using the Quick Change kit (Stratagene, La Jolla, CA), and mutant sequences were verified by dideoxy sequencing using Thermosequenase (Amersham Pharmacia Biotech).

Cell Culture and Transfection—COS-7 cells (American Type Culture Collection) were grown at 37 °C in a humidified atmosphere of 95% air, 5% CO_2, in DMEM supplemented with 10% FBS, 100 μg/ml streptomycin, and 100 units/ml penicillin (COS-7 medium). Cells were seeded at 8×10^5 cells/10-cm dish in COS-7 medium and cultured for 3 days prior to transfection using 5 ml/dish OptiMEM containing 10 μg/ml LipofectAMINE and the required pcDNA3.1 expression vectors for HA-AT_{1A}Rs (wild-type and DD mutants) alone (5 μg/100-mm dish) or together with the pcDNA I-$GRK2^{K220M}$ (3.75 and 2.5 μg DNA/100-mm dish, respectively) for 6 h at 37 °C. After changing to fresh COS-7 medium, the cells were cultured for another 2 days prior to use. Binding of ^{125}I-[Sar^1,Ile^8]Ang II to intact cells was determined as described previously (34).

HA-AT_{1A}-R Phosphorylation Assay—Transfected COS-7 cells in 10-cm dishes were metabolically labeled for 4 h at 37 °C in 5 ml of P_i-free DMEM containing 0.1% (w/v) BSA and 100 μCi/ml $^{32}P_i$. After three washes in KRH (118 mM NaCl, 2.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 10 mM glucose, 0.1% (w/v) BSA, 20 mM Hepes, pH 7.4), cells were incubated in the same medium for 10 min in a 37 °C water bath and Ang II (100 nM) was added for another 5 min. After three washes with ice-cold PBS, membrane lysates were prepared, extracted with salt/urea, and solubilized prior to deglycosylation with PNGase F as described (14, 15). Following immunoprecipitation with the HA.11 antibody and protein A-Sepharose, ^{32}P-labeled phospho-HA-AT_{1A}-Rs were eluted into sample buffer for 1 h at 48 °C and resolved by SDS-PAGE (8–16% gradient resolving gel) prior to visualization in a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). In order to quantify the relative phosphorylation of mutant HA-AT_{1A}-Rs, membrane lysates were normalized to an equal number of HA-AT_{1A}-Rs prior to immunoprecipitation. COS-7 cells from replicate 10-cm dishes were detached by trypsinization 24 h after transfection, reseeded into 24-well plates, cultured for another 24 h, and subjected to radioligand binding competition assay using ^{125}I-[Sar^1,Ile^8]Ang II. B_{max} values were obtained from Scatchard analysis of the binding data using the LIGAND program.

HA-AT_{1A}-R Internalization Assay—^{125}I-Ang II was added in serum-free Medium 199 containing 1 g/liter bovine serum albumin to transfected COS-7 cells in 24-well plates maintained at 37 °C for the required times. Incubations were stopped by rapid washing with ice-cold PBS, and acid-released and acid-resistant radioactivities were separated and measured by γ-spectrometry as described previously (35). The percentage of internalized ligand at each time point was calculated from the ratio of the acid-resistant specific binding to the total (acid-released + acid-resistant) specific binding. Nonspecific binding was determined in the presence of an excess (10 μM) of unlabeled [Sar^1,Ile^8]Ang II.

[^{3}H]Inositol Phosphate Measurements—Transfected COS-7 cells in 24-well plates were labeled by overnight incubation in inositol-free DMEM containing 0.1% (w/v) BSA, 2.5% (v/v) FBS, antibiotics, and 20 μCi/ml *myo*-[2-^{3}H]inositol. After washing and preincubation with 10 mM LiCl for 30 min, 100 nM Ang II was added for another 30 min. Inositol phosphates were extracted as described (36) and applied to Bio-Rad AG 1-X8 columns. After washing three times with water and twice with 0.2 M ammonium formate, 0.1 M formic acid, the combined [^{3}H]inositol bisphosphate + [^{3}H]inositol trisphosphate fractions were eluted with 1 M ammonium formate, 0.1 M formic acid, and radioactivities were determined by liquid scintillation counting. At the expression levels used in this study, there was a linear relationship between the number of cell surface receptors and the magnitude of agonist-stimulated [^{3}H]inositol phosphate production (37). Desensitization experiments were performed as described previously (13). Labeled COS-7 cells expressing the indicated receptors were preincubated in medium alone or with 100 nM Ang II for 3 min and washed with PBS before the treatment with 150 mM NaCl, 50 mM glycine, pH 3, for 30 s at 4 °C. After washing three times with ice-cold PBS, the cells were incubated for 15 min in medium containing 10 mM LiCl alone or with 100 nM Ang II. [^{3}H]Inositol phosphates were extracted and measured as described above.

Western Blotting—Transfected COS-7 cells in 10-cm dishes were grown for 1 day after transfection and reseeded in six-well plates. Cells were serum-starved for 24 h before treatment with 100 nM Ang II for the indicated time. After agonist stimulation at 37 °C, medium was aspirated and cells were washed twice with ice-cold PBS, then lysed in 100 μl of Laemmli sample buffer. The samples were briefly sonicated, heated at 95 °C for 5 min, and centrifuged for 5 min. The supernatants were electrophoresed on SDS-PAGE (8–16%) gradient gels and transferred to polyvinylidene difluoride nylon membranes. GRK2 or phosphorylated ERKl and ERK2 were detected by using a polyclonal rabbit anti-GRK2 antibody (0.5 μg/ml) (Santa Cruz Biotechnology, Santa Cruz, CA) or a monoclonal mouse phospho-ERK1/2-specific antibody (1:2000 dilution) (Cell Signaling Technology, Beverly, MA), respectively. Blots were probed with a 1:3000 dilution of anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibodies and visualized by using by ECL (enhanced chemiluminescence reagent), then quantified by scanning laser densitometry (Amersham Pharmacia Biotech).

RESULTS

Agonist-induced Phosphorylation of DD Mutants of the HA-AT_{1A}-Rs—Substitution of both residues of the diacidic motif (Asp^{236}-Asp^{237}) located at the carboxyl-terminal end of the third intracellular loop (31) with either alanine (DD-AA) or asparagine (DD-NN) reduced the expression of the mutant receptors below that of the wild-type AT_1 receptor (Table I). However, the K_d values of these and other relevant mutant receptors were similar to those of the wild-type receptor, demonstrating that the Asp^{236}-Asp^{237} motif is not required for high affinity ligand binding to the AT_{1A}-R. In subsequent studies, the relative degrees of agonist-induced phosphorylation of mutant receptors were determined using solubilized membrane lysates normalized to an equal number of receptors, as calculated from B_{max} values derived from radioligand binding assays in replicate transfected cells prior to immunoprecipitation.

The phosphorylated HA-AT_{1A}-R extracted from Ang II-stimulated COS-7 cells migrates as a diffuse band of M_r 85,000–145,000 in SDS-PAGE (14, 15). This broad migration pattern, which is largely due to variable degrees of receptor glycosylation and possibly the presence of comigrating phosphoproteins, interferes with the quantitation of the native phospho-HA-AT_{1A}-Rs. For this reason, the solubilized phosphoreceptors

TABLE I
Binding parameters of mutant HA-AT_{1A}-Rs

Intact COS-7 cells expressing the indicated receptors were subjected to radioligand binding competition assays for 2 h at 22 °C using ^{125}I-[Sar^1,Ile^8]Ang II, and K_d and B_{max} values were calculated using the LIGAND program. The B_{max} value for the wild-type (WT) receptor was 1.7 ± 0.4 pmol/mg of protein. The data represent mean values (± S.E.) from six to eight independent experiments.

Receptor	K_d	B_{max}
	nM	*% WT*
WT	1.6 ± 0.1	100
DD-AA	1.5 ± 0.2	52 ± 2
DD-NN	1.5 ± 0.2	61 ± 2
DD-AD	1.7 ± 0.2	83 ± 2
DD-DA	1.7 ± 0.2	40 ± 3
DD-ED	1.5 ± 0.1	94 ± 5
DD-DE	1.6 ± 0.1	88 ± 5
DD-EE	1.8 ± 0.2	81 ± 2
Δ335	1.6 ± 0.1	85 ± 4

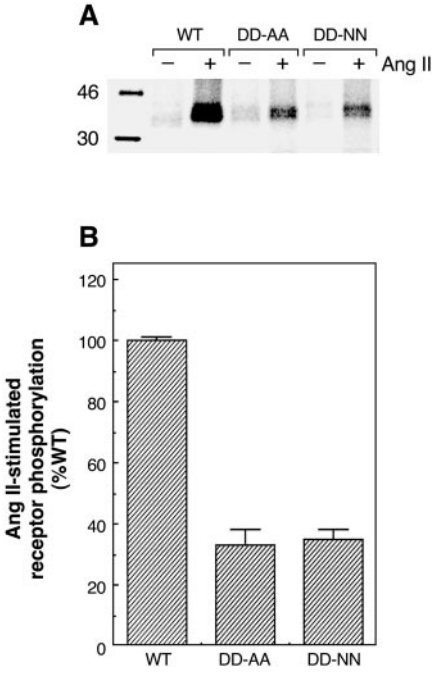

FIG. 1. **Agonist-induced phosphorylation of diacidic mutants of the HA-AT_{1A}-Rs.** *A*, COS-7 cells expressing the HA-AT_{1A} (*WT*) or the DD mutant (*DD-AA* and *DD-NN*) angiotensin receptors were labeled for 4 h with $^{32}P_i$ before stimulation with vehicle or 100 nM Ang II for 5 min. Membrane lysates were prepared, extracted with salt/urea, and solubilized prior to deglycosylation with PNGase F as described (13, 14). Following normalization of samples to an equal number of receptors, ^{32}P-labeled phospho-HA-AT_{1A}-Rs were immunoprecipitated with the anti-HA antibody, resolved by SDS-PAGE, and visualized in a PhosphorImager. *B*, quantification of five independent experiments are expressed as mean ± S.E. of the receptor phosphorylation induced by 100 nM Ang II. Receptor expression levels are shown in Table I.

were subjected to enzymatic deglycosylation with PNGase F prior to SDS-PAGE analysis. The deglycosylated phospho-HA-AT_{1A}-R runs as a discrete doublet of M_r ~40,000, consistent with the predicted molecular mass (41 kDa) of the receptor, and is separate from extraneous phosphoproteins (15). Under these conditions, minimal basal phosphorylation of the wild-type HA-AT_{1A}-R, or of the two DD mutant receptors, was observed in control cells (Fig. 1*A*). However, exposure of wild-type HA-AT_{1A}-R-expressing COS-7 cells to 100 nM Ang II for 5 min caused prominent phosphorylation of the receptor (Fig. 1*A*). In contrast, substitution of both Asp^{236} and Asp^{237} with alanine (DD-AA) or asparagine (DD-NN) reduced agonist-induced receptor phosphorylation by >60% (Fig. 1*B*).

Functional Characterization of Agonist-induced Signaling Responses of DD Mutant HA-AT_{1A}-Rs—In principle, mutation of Asp^{236} and/or Asp^{237} to alanine or asparagine might "lock" the HA-AT_{1A}-R into a conformation that is incapable of activation in response to agonist. Such inactive receptors would not only fail to isomerize to a conformation that permits phosphorylation, but could also be unable to transduce intracellular signals. However, exposure of DD-AA- or DD-NN-expressing COS-7 cells to Ang II caused a significant increase in [^{3}H]inositol phosphate production (Fig. 2*A*). Furthermore, when Ang II-stimulated increases in [^{3}H]inositol phosphate accumulation were normalized to an equal number of receptors, there was no reduction in the responses of the DD-AA- and DD-NN-expressing cells (Fig. 2*B*). In addition, Ang II-induced ERK activation, normalized to an equal degree of receptor expression, was also unaffected by the DD mutations. As shown in Fig. 2*C*, no differences in ERK phosphorylation were observed in COS-7 cells expressing wild-type or DD mutant AT_{1A} receptors after 5 min of stimulation with 100 nM Ang II. These data indicate that the agonist-stimulated DD-AA and DD-NN receptors can isomerize to an active conformation with full efficacy for coupling to $G_{q/11}$ signaling and activation of the ERK phosphorylation cascade. Furthermore, since the magnitudes of Ang II-stimulated [^{3}H]inositol phosphate and ERK responses mediated by the DD-AA and DD-NN receptors did not exceed that mediated by the wild-type HA-AT_{1A}-R, phosphorylation may not be essential for the desensitization mechanism(s) that normally limit signaling by the AT_1 receptor (15). In this context, we compared the magnitudes of Ang II-stimulated [^{3}H]inositol phosphate production mediated by the wild-type and the DD mutants with that mediated by a Δ335 truncated AT_1 receptor. As observed previously (15), Ang II-induced intracellular signaling by the truncated AT_{1A}-R was increased, an effect attributable to reduced desensitization of the hypophosphorylated receptor. The ability of the agonist-activated Δ335 receptor to stimulate inositol phosphate production was much greater than those of the wild-type and DD mutant receptors (Fig. 2, *A* and *B*), as was its effect on ERK signaling (data not shown).

Agonist-induced Desensitization of DD Mutant HA-AT_{1A}-Rs—The capacity of the mutant receptors to undergo agonist-induced desensitization was evaluated (13) by pretreatment for 3 min with 100 nM Ang II, which significantly reduced the subsequent maximal Ang II-stimulated inositol phosphate production as compared with non-treated control cells expressing the wild-type receptor (Fig. 3*A*). Attenuation of inositol phosphate generation was also observed when the DD mutant receptors were pretreated with 100 nM Ang II (Fig. 3*A*). Under similar conditions a small reduction was observed for the Δ335 receptor and was insignificant after normalization for receptor expression (Fig. 3*B*).

Internalization of DD Mutant HA-AT_{1A}-Rs—In view of the apparent difference in the role that phosphorylation plays in the function of the HA-AT_{1A}-R compared with that of the β_2-AR, we also examined the internalization kinetics of the DD-AA and DD-NN receptors in COS-7 cells. Like the [^{3}H]inositol phosphate and ERK responses mediated by these receptors, there was little difference in the rate and magnitude of ^{125}I-Ang II internalization in DD-AA- or DD-NN-expressing cells compared with those expressing the wild-type receptor (Fig. 4). Hence, in contrast to the β_2-AR, the majority of the agonist-induced HA-AT_{1A}-R phosphorylation does not appear to be required for its internalization following Ang II stimulation.

Structural Requirements of the DD Motif for Optimal Agonist-induced Phosphorylation, Inositol Phosphate Signaling, and Receptor Internalization—An evaluation of the structural requirements of the Asp^{236}-Asp^{237} motif for optimal AT_{1A}-R

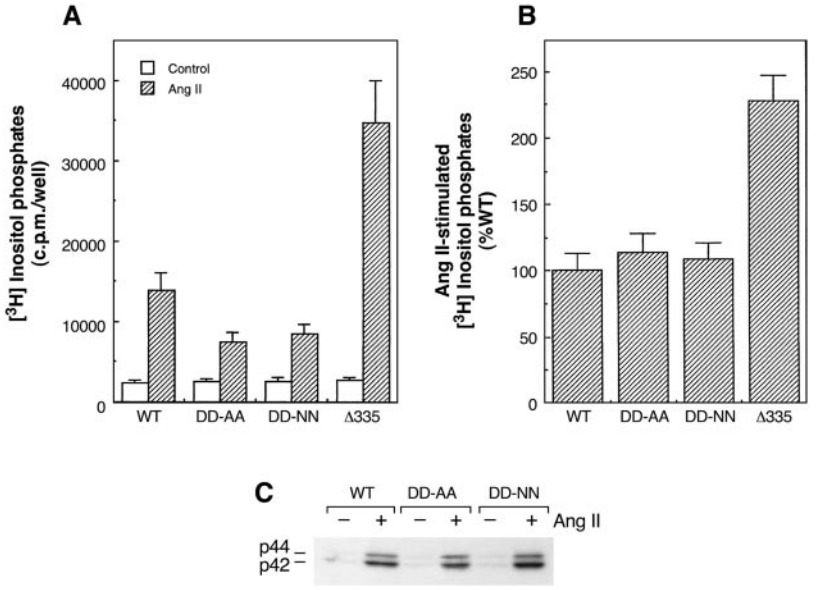

FIG. 2. **Inositol phosphate responses and mitogen-activated protein kinase activation by mutant HA-AT_{1A}-Rs.** *A* and *B*, [^{3}H]inositol-labeled COS-7 cells expressing the indicated receptors were preincubated with 10 mM LiCl for 30 min prior to the addition of vehicle or 100 nM Ang II for an additional 30 min. [^{3}H]Inositol phosphates were extracted and measured as described under "Experimental Procedures." *A*, mean (± S.E.) basal and Ang II-stimulated [^{3}H] inositol phosphate values from four independent experiments. *WT*, wild-type. *B*, inositol phosphate responses normalized for receptor expression and shown as percentage of the wild-type response. The Ang II-stimulated data are normalized to an equal number of receptors. *C*, ERK1/2 activation in serum-starved COS-7 cells transiently transfected with the indicated receptors and stimulated with 100 nM Ang II for 5 min.

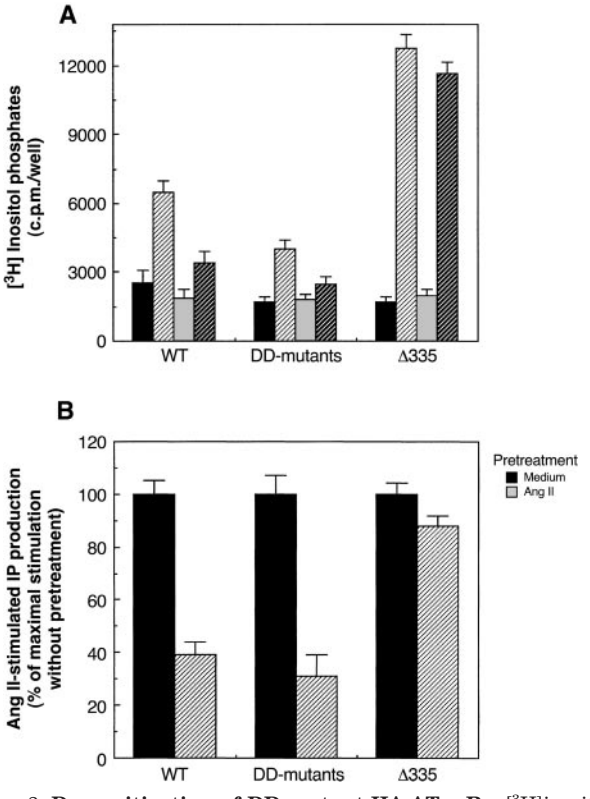

FIG. 3. **Desensitization of DD mutant HA-AT_{1A}-Rs.** [^{3}H]inositol-labeled COS-7 cells expressing the indicated receptors were preincubated in medium alone (*first two columns* of the indicated receptor) or with 100 nM Ang II (*third* and *fourth columns*) for 3 min and treated with 150 mM NaCl, 50 mM glycine, pH 3, for 30 s, and washed three times with PBS. The cells were then incubated for 15 min in medium containing 10 mM LiCl alone (*solid bars*) or with 100 nM Ang II (*hatched bars*). [^{3}H]Inositol phosphates were extracted and measured. *A*, mean (± S.E.) basal and Ang II-stimulated [^{3}H]inositol phosphate values from four independent experiments. *WT*, wild-type. *B*, data normalized to an equal number of receptors and expressed as percentage of maximal stimulation in the absence of Ang II pretreatment.

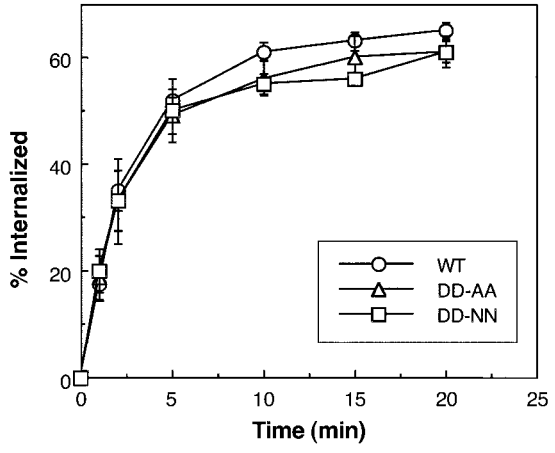

FIG. 4. **Internalization kinetics of DD mutant HA-AT_{1A}-Rs.** COS-7 cells expressing the HA-AT_{1A} (*WT*) or the DD mutant (*DD-AA* and *DD-NN*) angiotensin receptors were incubated with ^{125}I-Ang II at 37 °C for the indicated times. Acid-resistant and acid-sensitive binding (cpm) were determined as described under "Experimental Procedures," and the specific internalized (acid-resistant) binding was expressed as a percentage of the total binding at each time point. The data in each panel represent mean values (± S.E.) from three independent experiments.

phosphorylation and signaling responses showed that substitution of either acidic residue with alanine (DD-AD, DD-DA) or glutamic acid (DD-DE, DD-ED) had little effect on Ang II-induced AT_{1A}-R phosphorylation (Fig. 5*A*). Replacement of both Asp^{236} and Asp^{237} with glutamic acid residues (DD-EE) likewise did not impair Ang II-induced HA-AT_{1A}-R phosphorylation. Consistent with the normal signaling of the DD-AA and DD-NN receptors (Fig. 2*A*), the [^{3}H]inositol phosphate responses mediated by the DD-AD, DD-DA, DD-DE, DD-ED, and DD-EE receptors were not significantly reduced (Fig. 5*B*). Similarly, the rate and magnitude of ^{125}I-Ang II internalization by the mutant receptors were the same as those of the wild-type receptor (Fig. 5*C*). Taken together, these data indicate that a single acidic residue at position 236 or 237 is necessary for optimal phosphorylation of the AT_{1A}-R. Substitution of either Asp^{236} or Asp^{237} with alanine or glutamic acid had no effect on agonist-induced receptor phosphorylation. In contrast, substitution of both Asp^{236} and Asp^{237} with asparagine or alanine residues progressively reduced the receptor phosphorylation, which was minimal for the DD-AA receptor. These findings suggest that the majority of agonist-induced HA-AT_{1A}-R phosphorylation in COS-7 cells is mediated by an acidotropic receptor kinase, consistent with the participation of one or more GRKs in this process.

Effects of $GRK2^{K220M}$ and Bisindolylmaleimide on Agonist-induced Phosphorylation of DD Mutant HA-AT_{1A}-Rs—Previous

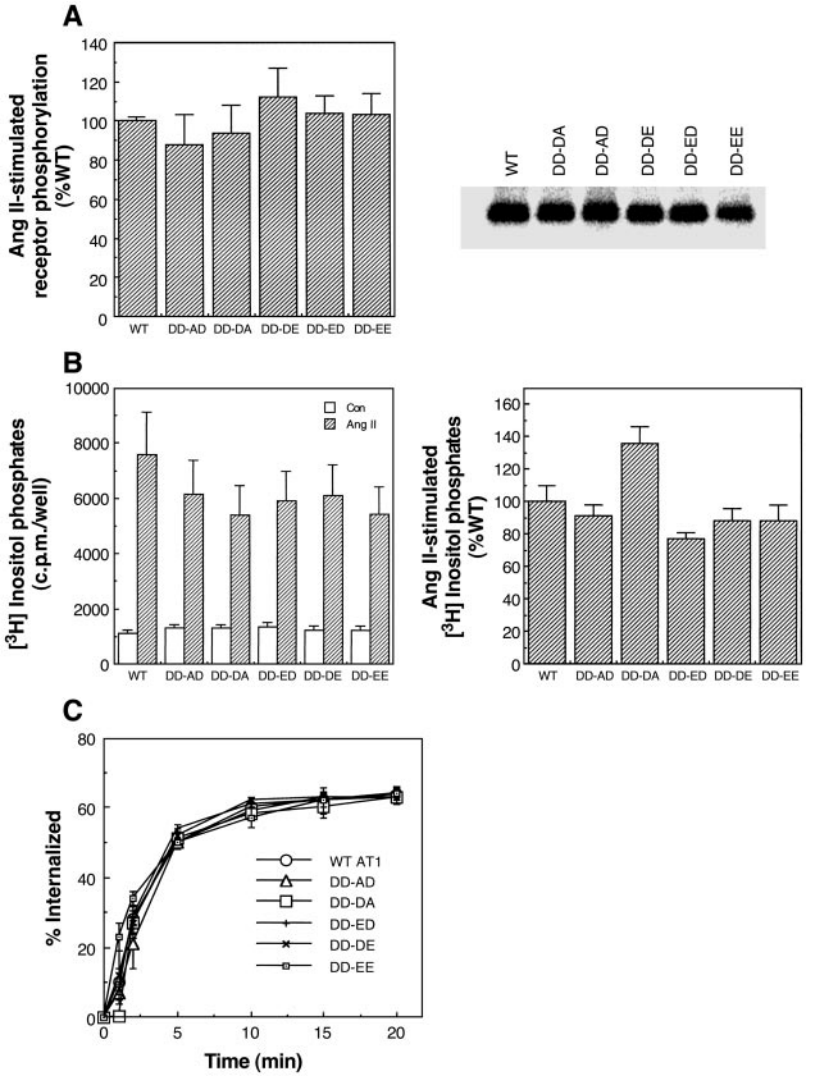

FIG. 5. **Structural requirements of the Asp^{236}-Asp^{237} motif for optimal agonist-induced phosphorylation, inositol phosphate response, and internalization kinetics of the HA-AT_{1A}-Rs.** *A*, receptor phosphorylation in COS-7 cells expressing the indicated mutants after stimulation with 100 nM Ang II for 5 min, expressed as percentage of the Ang II-induced receptor phosphorylation in control cells. A representative image is shown at *right*. *WT*, wild-type. *B*, [^{3}H]Inositol phosphate production in COS-7 cells expressing the indicated receptors during stimulation with 100 nM Ang II for 30 min. Mean (± S.E.) basal and Ang II-stimulated [^{3}H]inositol phosphates values from three independent experiments are shown in the *left panel*. Data are shown as percentage of the wild-type response in the *right panel*. *C*, agonist-induced endocytosis in COS-7 cells expressing the indicated receptors during incubation with ^{125}I-Ang II at 37 °C for up to 20 min. Acid-resistant and acid-sensitive radioligand binding (cpm) were determined as described under "Experimental Procedures." In *A* and *B* (*right panel*), the Ang II-stimulated data are normalized to an equal number of receptors.

studies have indicated that the consensus sequence for GRK-mediated phosphorylation of GPCRs includes acidic amino acids adjacent to the phosphorylation sites, whether located in the third intracellular loop (22, 23, 27, 28) or the cytoplasmic tail (19, 29, 30). We (15) and others (16) have observed previously that agonist-induced phosphorylation of the AT_{1A} receptor is mainly localized to a serine/threonine-rich region (Ser^{326}–Ser^{338}) of its carboxyl-terminal tail. However, the three acidic residues (at Asp^{343}, Glu^{357}, and Glu^{359}) (15) in the cytoplasmic tail do not conform to potential consensus sites for GRK-mediated phosphorylation of the HA-AT_{1A}-R. The only potentially relevant acidic motif located within the cytoplasmic regions of the AT_{1A}-R is the Asp^{236}/Asp^{237} sequence in the third intracellular loop. To determine whether this motif is required for GRK-mediated phosphorylation of the agonist-activated AT_{1A}-R, we coexpressed a dominant negative mutant GRK2 ($GRK2^{K220M}$) with the HA-AT_{1A}-R or its DD mutants in COS-7 cells. $GRK2^{K220M}$ retains the ability to bind to the agonist-occupied receptor, but is devoid of catalytic activity and acts as a competitive inhibitor of GRK2 activity (33, 38, 39). Overexpression of a related mutant GRK2 ($GRK2^{K220R}$) inhibits agonist-stimulated phosphorylation of the α_{1B}-adrenergic receptor transiently expressed in COS-7 cells (39), suggesting the endogenous expression of GRK2 or a related kinase in this cell line. In the present study, transfection of $GRK2^{K220M}$ with the HA-AT_{1A}-R caused a 30–40-fold overexpression of the mutant kinase compared with endogenous GRK2 levels in untransfected COS-7 cells (Fig. 6*A*). When ^{32}P-labeled cells expressing the HA-AT_{1A}-R were transfected with $GRK2^{K220M}$, agonist-induced phosphorylation was reduced by ~40% due to the inhibitory effect of the mutant kinase on the binding of its endogenous counterpart to the agonist-activated receptor (Fig. 6*A*). These results are consistent with a previous report in which agonist-induced AT_1-R phosphorylation was reduced by 50% in transfected HEK 293 cells overexpressing $GRK2^{K220R}$ (13).

In contrast to its prominent effect on the native AT_1-R, coexpression of $GRK2^{K220M}$ did not further decrease agonist-stimulated phosphorylation of the DD mutants (Fig. 6*A*). This finding is consistent with the involvement of GRK2 or a similar acidotropic receptor kinase in agonist-induced HA-AT_{1A}-receptor phosphorylation in COS-7 cells. In addition, the requirement for the Asp^{236}/Asp^{237} motif at the carboxyl-terminal end of the third intracellular loop for optimal agonist-induced receptor phosphorylation of the HA-AT_{1A}-R suggests that the proximal acidic environment necessary for GRK action must be conformationally adjacent to the region of the carboxyl-terminal tail that contains the phosphorylation sites (15, 16).

The participation of other kinase(s) that do not require the presence of the diacidic motif in agonist-induced phosphorylation was examined in studies on the effect of PKC inhibition by bisindolylmaleimide (BIM) on Ang II-induced phosphorylation. As shown in Fig. 6*B*, the action of Ang II on HA-AT_{1A} and DD mutant receptor phosphorylation was reduced by ~30% by

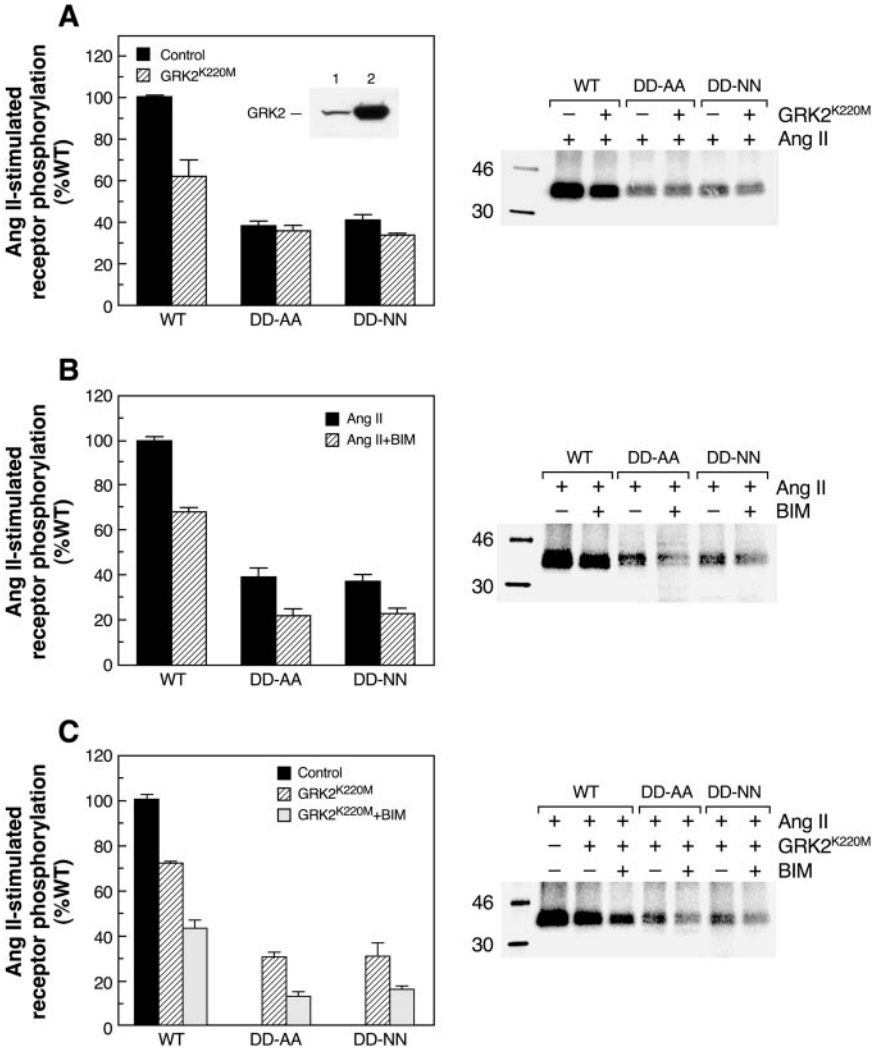

FIG. 6. **Effects of dominant negative GRK2 and BIM on agonist-induced phosphorylation of mutant HA-AT_{1A}-Rs.** COS-7 cells were transfected with the DNAs encoding the HA-AT_1-R or the DD mutants alone (*panel A* (*Control*) and *panel B*) or in combination with DNA encoding dominant negative mutant $GRK2^{K220M}$ (*panels A* and *C*, $GRK2^{K220M}$). *A*, ^{32}P-labeled COS-7 cells were stimulated with 100 nM Ang II. The *inset* shows a representative Western blot of lysates prepared from cells transfected with HA-AT_{1A} (*lane 1*) or HA-AT_1 plus $GRK2^{K220M}$ (*lane 2*) and immunoblotted with polyclonal anti-GRK2 antibody. *WT*, wild-type. *B* and *C*, ^{32}P-labeled COS-7 cells were preincubated in the presence or absence of 1 μM BIM for 30 min and stimulated for 5 min with 100 nM Ang II. Data are expressed as percentage of Ang II-induced responses in wild-type cells. The means and ± S.E. of three to four experiments using different cell preparations are shown. Representative autoradiographs are shown at the *right*.

BIM, consistent with a previous study in hepatic C-9 cells (40). An additive inhibitory effect of $GRK2^{K220M}$ and BIM was observed on agonist-stimulated HA-AT_{1A}-R phosphorylation (Fig. 6*C*). However, the combined inhibitory effect of both $GRK2^{K220M}$ and BIM on the agonist-induced receptor phosphorylation of DD mutant HA-AT_{1A}-Rs was similar to that of BIM alone (Fig. 6, *B* and *C*). This result indicates the participation of PKC as well as GRK2 and possibly other kinases in agonist-mediated phosphorylation. Indeed, several reports have shown that PKC has an important role in homologous and heterologous receptor phosphorylation and desensitization (40–43).

Effect of $GRK2^{K220M}$ on Agonist-induced Internalization of DD Mutant HA-AT_{1A}-Rs—The relationship between agonist-induced receptor phosphorylation and endocytosis was investigated by analyzing the rate and the extent of receptor internalization in cells expressing the HA-AT_{1A}-R alone or coexpressing $GRK2^{K220M}$. Internalization was unaltered by cotranfection of cells with the dominant-negative GRK2, indicating that endocytosis of the HA-AT_{1A} receptor is mediated by mechanisms distinct from its GRK2-dependent phosphorylation (Fig. 7*A*). Similar results were obtained in cells coexpressing the DD mutants and $GRK2^{K220M}$ (Fig. 7, *B* and *C*).

DISCUSSION

Our results indicate that the diacidic motif required for COOH-terminal phosphorylation of the HA-AT_{1A}-R is located on a separate domain, in the third intracellular loop (15). Furthermore, in contrast to the proposed diacidic motifs required for phosphorylation of rhodopsin and the β_2-AR (Asp-Glu and Glu-Asp, respectively) (19), only a single acidic residue is required for optimal HA-AT_{1A}-R phosphorylation. However, since the Asp-Glu and Glu-Asp motifs each contain an aspartic acid residue, and no additional structural requirements have been reported for these motifs, a single aspartic acid residue may be sufficient for optimal phosphorylation of rhodopsin and the β_2-AR. It should be noted that the preference of GRKs for acidic residues immediately adjacent to the phosphorylated moiety was established using synthetic peptides of ~12 amino acids that lack the complex structure of an intact receptor. The present study has demonstrated that the intracellular regions of the AT_{1A} receptor contain functionally important acidic residues (Asp^{236}-Asp^{237} in the distal portion of the third intracellular loop) that are separate from the identified sites of agonist-induced phosphorylation in the primary sequence (segment Ser^{326}–Thr^{338} of the receptor's cytoplasmic tail; Refs. 15 and 16). Nevertheless, these residues can apparently provide an acidic environment for GRKs within the context of the agonist-induced receptor conformation.

Interestingly, Small *et al.* (28) have recently described the importance of an acidic motif in the normal regulation of the α_{2B}-adrenergic receptor. A polymorphic variant of the human α_{2B}-adrenergic receptor, with a deletion of three glutamic acids in the third intracellular loop, exhibited normal ligand binding but showed decreased agonist-induced phosphorylation mediated by GRKs and complete loss of short term receptor desen-

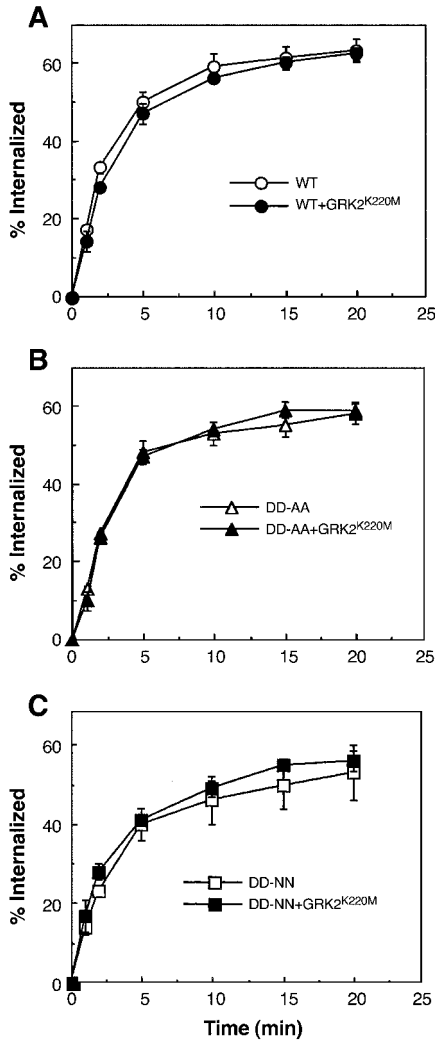

FIG. 7. **Lack of effect of dominant negative GRK2 on agonist-induced internalization of ^{125}I-Ang II in wild-type (*WT*) and DD mutant receptors.** *A*, COS-7 cells expressing the HA-AT_1 (*A*) or the DD mutant (*B* and *C*) receptors alone or in conjunction with dominant negative GRK2 were incubated with ^{125}I-Ang II at 37 °C for the indicated times. Acid-resistant and acid-sensitive binding (cpm) were determined as described under "Experimental Procedures," and the specific internalized (acid-resistant) binding was expressed as a percentage of the total binding at each time point. The data in each panel represent mean values (± S.E.) from three independent experiments.

sitization. This deletion occurs within a stretch of acidic residues that appears to establish an optimal milieu for GRK function (23, 28), supporting the importance of acidic residues in the structural motif for GRK phosphorylation.

In addition to these variations in the structural requirements for receptor phosphorylation, it is evident that a major difference exists in the role of phosphorylation in the functions of the β_2-AR and the AT_{1A}-R. Whereas GRK-mediated phosphorylation is a critical factor in the desensitization of G_s-mediated signaling via the β_2-AR (1–4), it apparently has no major role in the desensitization of G_q-mediated signaling via the HA-AT_{1A}-R in COS-7 cells. Likewise, although GRK-mediated phosphorylation is essential for agonist-induced internalization of the β_2-AR via clathrin-coated pits (1–4), it is not required for internalization of the AT_{1A}-R.

The presence in many GPCRs of serine/threonine-rich regions in their cytoplasmic tail or third intracellular loop suggests that phosphorylation of these regions regulates the internalization process. Several studies on the role of receptor phosphorylation in the regulation of GPCR sequestration have indicated that phosphorylation of GPCRs by GRKs, in addition to causing homologous desensitization, can influence their internalization (33, 44, 45). Co-expression of GRK2 with the G_i-coupled M2 muscarinic acetylcholine receptor facilitated, and that of dominant negative GRK2^{K220W} attenuated, receptor sequestration in COS-7 cells (44). Similar effects of GRK2 and GRK2^{K220M} were observed with the β_2-adrenergic receptor (33). A role of GRK-mediated phosphorylation in GPCR internalization is consistent with the proposed function of β-arrestins as adapter proteins for the endocytosis of these receptors (2). Additionally, GRK2 and β-arrestins have been shown to act synergistically to regulate β_2-adrenergic receptor internalization (45). However, several observations argue against a general role of receptor phosphorylation in the regulation of GPCR internalization. For example, phosphorylation of the secretin receptor by protein kinase A has been proposed to regulate its internalization kinetics (46). In addition, the role of GRK2-mediated phosphorylation in the internalization of the parathyroid hormone receptor has been questioned by the finding that mutant parathyroid hormone receptors undergo endocytosis in the absence of detectable agonist-induced phosphorylation (20). These observations, and similar data obtained with a truncated opioid receptor (47), do not support a general and essential role for receptor phosphorylation in the control of GPCR internalization.

Previous studies using site-directed mutagenesis of the rat AT_{1A}-R have indicated that the major site of AT_1-R phosphorylation is localized to its carboxyl-terminal tail (15, 16, 43), and that Ser^{335}-Thr^{336} are rapidly phosphorylated after receptor activation (15, 16). Since these residues are also important for internalization of the receptor, it is likely that their phosphorylation regulates the kinetics of AT_1-R activation. In this context, it is possible that the conformational change caused by the DD mutations does not affect agonist-mediated phosphorylation of Ser^{335}-Thr^{336}, since the mutant receptors exhibited normal internalization kinetics. Furthermore, mutations of Asp^{236} and Asp^{237} did not cause the marked increase in Ang II-induced inositol phosphate accumulation as observed for the double alanine substitution of Ser^{335} and Thr^{336} (15). These data suggest that, although GRK2 is not involved in the phosphorylation of the DD mutant receptor, the residual phosphorylation sites of this receptor include residues critical for internalization and desensitization of the receptor.

The role of AT_1-R phosphorylation in the internalization of the receptor was also questioned by the recent findings that constitutively active (N111A and N111G) mutant AT_{1A}-Rs show markedly impaired phosphorylation yet have normal internalization kinetics (48). However, it is not known whether the constitutively active AT_1-R utilizes the same internalization pathway as the wild-type receptor. The possibility that the DD mutant AT_1-Rs are constitutively active was excluded by the absence of increased basal inositol phosphate production (Fig. 2*A*), and the inability of partial agonists such as [Sar^1,Ile^8]Ang II, [Sar^1,Ile^4,Ile^8]Ang II, and CGP 42112A, which strongly activate signaling from constitutively active AT_1 receptors (48, 49), to stimulate inositol phosphate production by the DD mutant receptors (data not shown).

Agonist-induced phosphorylation of the AT_1-R has been reported to be mediated predominantly by PKC at low Ang II concentrations and by other kinases at higher agonist levels (41–43). However, although all three predicted PKC sites (Ser^{331}, Ser^{338}, and Ser^{348}) in the cytoplasmic tail are phosphorylated during activation of the rat AT_{1A}-R (43), these do not include most of the amino acids that are required for AT_1-R internalization (3, 15, 16). In agreement with this report, we have observed that inhibition of PKC by BIM does not affect

agonist-mediated internalization of the wild-type and the DD mutant receptors (data not shown). These findings suggest that, although PKC-induced phosphorylation promotes the desensitization of the AT_1-R signaling responses, it has little or no role in its agonist-induced internalization (41, 43).

Our observation that the diacidic motif at position 236–237 is required for optimal conformation of the receptor for GRK-mediated phosphorylation is consistent with the probability that the AT_{1A} receptor can assume multiple conformational states associated with the individual stages of receptor activation and regulation (48). The inability of the present AT_{1A} receptor mutants to assume the conformation required for phosphorylation, while retaining that required for inositol phosphate signaling and internalization, also argues against the assumption that the active, signaling state of GPCRs is the same as that recognized by GRKs and targeted for phosphorylation.

It has been demonstrated that internalization of the AT_{1A}-R in COS-7 cells is dynamin- and β-arrestin-dependent under our experimental conditions (50). It is possible that binding of β-arrestin to the activated receptor before its phosphorylation could explain the phosphorylation-independent internalization of constitutively active AT_1 receptors (48). However, in the case of the DD mutant AT_{1A}-Rs, it is more likely that the residual phosphorylation sites of the receptor, as noted above, include the residues critical for β-arrestin binding. The Ser^{335}-Thr^{336}-Leu^{337} sequence found previously to be essential for receptor internalization (15, 35) has been shown recently to be a major determinant of β-arrestin binding to the receptor (51). Since GRKs and PKC are unlikely to contribute to the phosphorylation that regulates internalization of the AT_{1A} receptor, other kinases should also be considered for this function. In this context, it is interesting that Tang *et al.* (42) reported the contribution of an unidentified heparin-sensitive kinase in agonist-induced phosphorylation and desensitization of the AT_1-R. Additionally, studies on the muscarinic receptor have suggested that casein kinase 1α participates in the agonist-induced phosphorylation of GPCRs (52).

The present data indicated that receptor kinases other than GRKs have a major role in the regulation of AT_1-R function. The specific role of Ang II-induced GRK phosphorylation in the regulation of AT_1-R function remains to be established. In addition to its actions in desensitization and internalization, receptor phosphorylation has been reported to switch the coupling of the β_2-AR from G_s to G_i (53). It is possible that phosphorylation of the AT_1-R may serve a similar purpose, especially since the latter receptor appears to couple to G_i as well as $G_{q/11}$ in several rodent cell types (40, 54). However, it is also possible that AT_1-R phosphorylation may serve some other, hitherto unrecognized, role in AT_1-R action. Further studies on the properties and functions of phosphorylation-deficient AT_{1A}-Rs and other GPCRs should clarify these issues.

Acknowledgments—We thank Yue Zheng and Judith Hernandez-Aranda for excellent technical assistance.

REFERENCES

1. Pitcher, J. A., Freedman, N. J., and Lefkowitz, R. J. (1998) *Annu. Rev. Biochem.* **67,** 653–692
2. Krupnick, J. G., and Benovic, J. L. (1998) *Annu. Rev. Pharmacol. Toxicol.* **38,** 289–319
3. Hunyady, L., Catt, K. J., Clark, A. J., and Gaborik, Z. (2000) *Regul. Pept.* **91,** 29–44
4. Ferguson, S. S. G. (2001) *Pharmacol. Rev.* **53,** 1–24
5. Haga, K., Kameyama, K., Haga, T., Kikkawa, U., Shiozaki, K., and Uchiyama, H. (1996) *J. Biol. Chem.* **271,** 2776–2782
6. Richardson, R. M., Kim, C., Benovic, J. L., and Hosey, M. M. (1993) *J. Biol. Chem.* **268,** 13650–13656
7. Kwatra, M. M., Schwinn, D. A., Schreurs, J., Blank, J. L., Kim, C. M., Benovic, J. L., Krause, J. E., Caron, M. J., and Lefkowitz, R. J. (1993) *J. Biol. Chem.* **268,** 9161–9164
8. Hipkin, R. W., Friedman, J., Clark, R. B., Eppler, M., and Schonbrunn, A. (1997) *J. Biol. Chem.* **272,** 13869–13876
9. Pei, G., Kieffer, B. L., Lefkowitz, R. J., and Freedman, N. J. (1995) *Mol. Pharmacol.* **48,** 173–177
10. Freedman, N. J., Ament, A. S., Oppermann, M., Stoffel, R. H., Exum, S. T., and Lefkowitz, R. J. (1997) *J. Biol. Chem.* **272,** 17734–17743
11. Palmer, T. M., Benovic, J. L., and Stiles, G. L. (1995) *J. Biol. Chem.* **270,** 29607–29613
12. Innamorati, G., Sadeghi, H., Eberle, A. N., and Birnbaumer, M. (1997) *J. Biol. Chem.* **272,** 2486–2492
13. Oppermann, M., Freedman, N. J., Alexander, R. W., and Lefkowitz, R. J. (1996) *J. Biol. Chem.* **271,** 13266–13272
14. Smith, R. D., Baukal, A. J., Zolyomi, A., Gaborik, Z., Hunyady, L., Sun, L., Zhang, M., Chen, H.-C., and Catt, K. J. (1998) *Mol. Endocrinol.* **12,** 634–644
15. Smith, R. D., Hunyady, L., Olivares-Reyes, J. A., Mihalik, B., Jayadev, S., and Catt, K. J. (1998) *Mol. Pharmacol.* **54,** 935–941
16. Thomas, W. G., Motel, T. J., Kule, C. E., Karoor, V., and Baker, K. M. (1998) *Mol. Endocrinol.* **12,** 1513–1524
17. Olivares-Reyes, J. A., Jayadev, S., Hunyady, L., Catt, K. J., and Smith, R. D. (2000) *Mol. Pharmacol.* **58,** 1156–1161
18. McDowell, J. H., Nawrocki, J. P., and Hargrave, P. A. (1993) *Biochemistry* **32,** 4968–4974
19. Fredericks, Z. L., Pitcher, J. A., and Lefkowitz, R. J. (1996) *J. Biol. Chem.* **271,** 13796–13803
20. Malecz, N., Bambino, T., Bencsik, M., and Nissenson, R. A. (1998) *Mol. Endocrinol.* **12,** 1846–1856
21. Nakata, H., Kameyama, K., Haga, K., and Haga, T. (1994) *Eur. J. Biochem.* **220,** 29–36
22. Eason, M. G., Moreira, S. P., and Liggett, S. B. (1995) *J. Biol. Chem.* **270,** 4681–4688
23. Jewell-Motz, E. A., and Liggett, S. B. (1995) *Biochemistry* **34,** 11946–11953
24. Pearson, R. B., and Kemp, B. E. (1991) *Methods Enzymol.* **200,** 62–81
25. Palczewski, K., Arendt, A., McDowell, J. H., and Hargrave, P. A. (1989) *Biochemistry* **28,** 8764–8770
26. Onorato, J. J., Palczewski, K., Regan, J. W., Caron, M. G., Lefkowitz, R. J., and Benovic, J. L. (1991) *Biochemistry* **30,** 5118–5125
27. Lee, K. B., Ptasienski, J. A., Bünemann, M., and Hosey, M. M. (2000) *J. Biol. Chem.* **275,** 35767–35777
28. Small, K. M., Brown, K. M., Forbes, S. L., and Ligget, S. B. (2001) *J. Biol. Chem.* **276,** 4917–4922
29. Pak, Y., O'Dowd, B. F., and George, S. R. (1997) *J. Biol. Chem.* **272,** 24961–24965
30. Deng, H. B., Yu, Y., Pak, Y., O'Dowd, B. F., George, S. R., Surratt, C. K., Uhl, G. R., and Wang, J. B. (2000) *Biochemistry* **39,** 5492–5499
31. Murphy, T. J., Alexander, R. W., Griendling, K. K., Runge, M. S., and Bernstein, K. E. (1991) *Nature* **351,** 233–236
32. Zhang, M., Zhao, X., Chen, H.-C., Catt, K. J., and Hunyady, L. (2000) *J. Biol. Chem.* **275,** 15782–15788
33. Ferguson, S. S. G., Ménard, L., Barak, L. S., Koch, W. J., Colapietro, A. M., and Caron, M. G. (1995) *J. Biol. Chem.* **270,** 24782–24789
34. Hunyady, L., Zhang, M., Jagadeesh, G., Bor, M., Balla, T., and Catt, K. J. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 10040–10045
35. Hunyady, L., Bor, M., Balla, T., and Catt, K. J. (1994) *J. Biol. Chem.* **269,** 31378–31382
36. Hunyady, L., Ji, H., Jagadeesh, G., Zhang, M., Gaborik, Z., Mihalik, B., and Catt, K. J. (1998) *Mol. Pharmacol.* **54,** 427–434
37. Hunyady, L., Bor, M., Baukal, A. J., Balla, T., and Catt, K. J. (1995) *J. Biol. Chem.* **270,** 16602–16609
38. Kong, G., Penn, R., and Benovic, J. L. (1994) *J. Biol. Chem.* **269,** 13084–13087
39. Diviani, D., Lattion, A.-L., Larbi, N., Kunapuli, P., Pronin, A., Benovic, J. L., and Cotecchia, S. (1996) *J. Biol. Chem.* **271,** 5049–5058
40. García-Caballero, A., Olivares-Reyes, J. A., Catt, K. J., and García-Saínz, J. A. (2001) *Mol. Pharmacol.* **59,** 576–585
41. Balmforth, A. J., Shepherd, F. H., Warburton, P., and Ball, S. G. (1997) *Br. J. Pharmacol.* **122,** 1469–1477
42. Tang, H., Guo, D. F., Porter, J. P., Wanaka, Y., and Inagami, T. (1998) *Circ. Res.* **82,** 523–531
43. Quian, H., Pipolo, L., and Thomas, W. G. (1999) *Biochem. J.* **343,** 637–644
44. Tsuga, H., Kameyama, K., Haga, T., Kurose, H., and Nagao, T. (1994) *J. Biol. Chem.* **269,** 32522–32527
45. Ménard, L., Ferguson, S. S. G., Zhang, J., Lin, F. T., Lefkowitz, R. J., Caron, M. G., and Barak, L. S. (1997) *Mol. Pharmacol.* **51,** 800–808
46. Walker, J. K., Premont, R. T., Barak, L. S., Caron, M. G., and Shetzline, M. A. (1999) *J. Biol. Chem.* **274,** 31515–31523
47. Murray, S. R., Evans, C. J., and Von Zastrow, M. (1998) *J. Biol. Chem.* **273,** 24987–24991
48. Thomas, W. G., Quian, H., Chang, C.-S., and Karnik, S. (2000) *J. Biol. Chem.* **275,** 2893–2900
49. Groblewski, T., Maigret, B., Larguier, R., Lombard, C., Bonnafous, J.-C., and Marie, J. (1997) *J. Biol. Chem.* **272,** 1822–1826
50. Gáborik, Z., Szaszák, M., Szidonya, L., Balla, B., Paku, S., Catt, K. J., Clark, A. J. L., and Hunyady, L. (2001) *Mol. Pharmacol.* **59,** 239–247
51. Oakley, R. H., Laporte, S. A., Holt, J. A., Barak, L. S., and Caron, M. G. (2001) *J. Biol. Chem.* **276,** 19452–19460
52. Budd, D. C., McDonald, J. E., and Tobin, A. B. (2000) *J. Biol. Chem.* **275,** 19667–19675
53. Daaka, Y., Luttrell, L. M., and Lefkowitz, R. J. (1997) *Nature* **390,** 88–91
54. De Gasparo, M., Catt, K. J., Inagami, T., Wright, J. W., and Unger, T. (2000) *Pharmacol. Rev.* **52,** 415–472